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Xanthohumol Suppresses Mylip/Idol Gene Expression and Modulates LDLR Abundance and Activity in HepG2 Cells

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Supporting Information

ABSTRACT: Xanthohumol, a prenylated flavonoid found in hops (*Humulus lupulus* L.), exhibits multiple biological activities such as antiatherosclerosis and hypolipidemic activities. In this study, we aim to investigate the hypocholesterolemic effects and molecular mechanisms of xanthohumol in hepatic cells. We found that xanthohumol (10 and 20 μM) increased the amount of cell-surface low-density lipoprotein receptor (LDLR) from 100.0 ± 2.1% to 115.0 ± 1.3% and 135.2 ± 2.7%, and enhanced the LDL uptake activity from 100.0 ± 0.9% to 139.1 ± 13.2% in HepG2 cells ($p < 0.01$). The mRNA levels of LDLR, HMGCR, and PCSK9 were not altered. Xanthohumol (20 μM) reduced the expression of inducible degrader of the LDL receptor (Mylip/Idol) mRNA and protein by approximately 45% ($p < 0.01$), which was reported to be associated with increases of LDLR level. We demonstrated that xanthohumol suppressed hepatic Mylip/Idol expression via counteracting liver X receptor (LXR) activation. The molecular docking results predicted that xanthohumol has a high binding affinity to interact with the LXRα ligand-binding domain, which may result in attenuation of LXRα-induced Mylip/Idol expression. Finally, we demonstrated that the Mylip/Idol expression and LDLR activity were synergistically changed by a combination of xanthohumol and simvastatin treatment. Our findings indicated that xanthohumol may regulate the LXR−Mylip/Idol axis to modulate hepatic LDLR abundance and activity.

KEYWORDS: *xanthohumol, LDLR, Mylip/Idol, LXRα, simvastatin*

INTRODUCTION

Patients with hypercholesterolemia present high levels of low-density lipoprotein cholesterol (LDL-C) in circulation and have a high risk for developing atherosclerosis and coronary heart disease (CHD).[1] The reduction of plasma LDL-C is known to decrease mortality of CHD patients.[2] Low-density lipoprotein receptor (LDLR) is a cell surface receptor that is expressed mainly in the liver and removes LDL particles from plasma by receptor-mediated endocytosis.[3] Deficiency in LDLR function has been reported to induce high concentrations of plasma LDL-C, familial hypercholesterolemia, and premature atherosclerosis.[4,5] Upregulation of LDLR activity in hepatocytes leads to enhanced LDL clearance from the circulation, thereby reducing the risk of atherogenic development and cardiovascular disorders. Therefore, the activity of hepatic LDLR is a major determinant of cholesterol levels in the plasma and may serve as a critical drug target for prevention or therapy of hypercholesterolemia.[6]

The expression of hepatic LDLR is predominantly regulated by sterol regulatory element binding protein-2 (SREBP-2), which binds to the sterol regulatory element (SRE) region of the LDLR promoter and promotes mRNA transcription, thereby increasing cell surface LDLR on hepatic cells.[7] In addition to transcriptional regulation, the abundance of hepatic LDLR is also controlled by post-transcriptional mechanisms. Recently, two major post-translational regulators of LDLR have been identified, including proprotein convertase subtilisin/kexin type 9 (PCSK9)[8] and myosin regulatory light chain-interacting protein (Mylip)/inducible degrader of the LDL receptor (Idol) (also known as Mylip/Idol).[9] PCSK9 and Mylip/Idol proteins enhance LDLR degradation, which impedes LDL clearance from plasma.[10−13]

PCSK9 is a plasma protein mainly produced in and secreted from the liver. PCSK9 binds to an extracellular domain of LDLR in hepatic cells for internalization, which impedes LDLR recycling to the cell surface and enhances trafficking to lysosomes.[14,15] The PCSK9 promoter contains a functional SRE that responds to intracellular cholesterol levels and is also mainly activated by the SREBP-2 transcription factor.[16] Several studies have shown that overexpression of PCSK9 reduces LDLR protein levels and increases the risk of hypercholesterolemia. The Mylip/Idol protein is a key regulator newly identified in the modulation of LDLR abundance.[9,12] The Mylip/Idol is an intracellular protein and has two functional domains, including an N-terminal FERM and a C-terminal RING domain.[17] The FERM domain of Mylip/Idol is a protein interaction motif that can specifically bind to the LDLR. The RING domain contains E3-ubiquitin ligase activity, which conjugates ubiquitin to the cytoplasmic tail of LDLR for protein degradation. Overexpression of Mylip/Idol protein has been reported to decrease LDLR abundance and attenuate

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LDL uptake activity in hepatic cells.[10,18] Distinct from the regulation of PCSK9 expression by SREBP, Mylip/Idol gene expression is induced by liver X receptors (LXRs), the critical nuclear receptors for gene transcription in cholesterol and lipid metabolism.[12] Activation of LXR signaling pathways may result in producing more Mylip/Idol protein in the liver, which decreases the amount of LDLR protein and increases LDL in plasma. Mylip/Idol reduction through inhibition of LXR activation has been reported to elevate LDLR levels and its activity.[19−21] Therefore, Mylip/Idol may serve as a novel target to develop cholesterol-lowering agents for lipid management.

Xanthohumol is the major prenylated flavonoid found in the female inflorescences of hops (*Humulus lupulus* L.). Xanthohumol has been reported to possess multiple biological activities, including anti-inflammation, anticancer, antiangiogenesis, and neuroprotection activities.[22−26] Recent studies have demonstrated that xanthohumol plays critical roles in the modulation of lipid homeostasis and metabolic syndromes. Xanthohumol has been shown to decrease triglyceride synthesis and apolipoprotein B secretion in a HepG2 cell model.[27] In 3T3-L1 adipocytes, xanthohumol reduces lipid content and adipogenesis.[28] Animal studies have shown that xanthohumol improves lipid and glucose metabolism in obesity and type 2 diabetes mellitus mouse models.[29] Xanthohumol may prevent atherosclerosis by decreasing arterial cholesterol and by increasing HDL cholesterol in the cholesterol ester transfer protein transgenic mouse (CETP-Tg) model.[30] Xanthohumol may lower body weight and modulate dysfunctional glucose and lipid metabolisms as well as reduce plasma cholesterol concentration and hepatic steatosis in obese animal models or ApoE-deficient mice.[31−33] These studies have shown that xanthohumol exerts lipid-modulating effects in vivo, but detailed mechanisms underlying these effects remain to be elucidated.

The aim of this study was to investigate the hypocholesterolemic effects of xanthohumol and underlying molecular mechanisms in HepG2 cells. We focused on the effects of xanthohumol on LDLR expression, LDL uptake, and Mylip/Idol gene expression in HepG2 cells. We also investigated the effect of combining xanthohumol and statin on LDLR activity in HepG2 cells.

MATERIALS AND METHODS

Chemicals. Xanthohumol was purchased from Enzo Life Science (Ann Arbor, MI, USA). Simvastatin was purchased from Tokyo Chemical Industry (Toshima, Kita-Ku, Tokyo, Japan). Dimethyl sulfoxide (DMSO), nonessential amino acids (NEAAs), T0901317, and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise indicated. Gibco fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific, Inc. (Rockford, IL, USA).

Cell Culture and Compound Treatments. The HepG2 cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cell line was maintained in DMEM medium containing 10% fetal bovine serum (FBS) and 1× nonessential amino acid (NEAA) solution, and it was incubated at 37 °C in a 5% CO_2 incubator. The cells (1×10^6 cells/mL) were seeded in culture medium overnight, and the medium was then changed to DMEM supplemented with 5% lipoprotein-deficient serum (LPDS) for 24 h incubation. Cells were treated with vehicle (0.1% DMSO) or xanthohumol for an additional 24 h. For T0901317 LXR agonist or simvastatin treatment, HepG2 cells were pretreated with the vehicle or xanthohumol for 1 h followed by incubation with T0901317 (1 or 2 μM) or simvastatin (1 μM) for an additional 24 h.

Analysis of Cell Viability by MTT Assay. Cells were seeded in 24-well plates as described above and treated with vehicle or xanthohumol (5−40 μM) for 24 h. After incubation, MTT reagent (1 mg/mL) was added to each well and incubated at 37 °C for 3 h. The medium was removed, and the purple formazan crystals were dissolved in DMSO. Absorbance was measured at 550 nm using a microplate reader.

Western Blot Analysis. Cells were seeded as described above and treated with vehicle or xanthohumol for 24 h. Total cellular proteins were harvested using RIPA buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA). For nuclear extract preparation, cells were harvested using the NE-PER nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific). The protein samples were separated on 10% SDS−PAGE and transferred to PVDF membranes (PerkinElmer, Boston, MA, USA). The membranes were incubated with the following specific antibodies: anti-MYLIP (#ab134994, Abcam, Cambridge, U.K.), anti-LXRα (Abcam), anti-LDLR (Novus Biologicals, Littleton, CO, USA), anti-β-actin (Sigma-Aldrich), and anti-PCSK9 and anti-HDAC2 (GeneTex, Irvine, CA, USA). The membranes were incubated with the appropriate HRP-conjugated secondary antibodies, and the proteins were detected using Amersham ECL Prime Western Blotting Detection Reagent. The chemiluminescent signal was visualized using Amersham Hyperfilm ECL film (GE Healthcare, Buckinghamshire, U.K.).

Flow Cytometric Analysis of Cell-Surface LDLR. The amount of cell surface LDLR was measured by flow cytometric analysis as previously described.[34] Briefly, cells were seeded as described above, and cells were then incubated with DMEM containing 5% LPDS for 24 h. Cells were then treated with vehicle or xanthohumol (10 and 20 μM) for an additional 24 h. Cells were detached by scraping, washed with PBS, and incubated with PBS containing 5% bovine serum albumin at room temperature for 30 min. After blocking, the cells were incubated with the anti-LDLR antibody at 37 °C for 1 h, washed with PBS, and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) at room temperature for 30 min. Cells were resuspended in PBS and analyzed by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA). Data were calculated using Cell Quest Pro software (BD Biosciences), and the cell surface LDLR was expressed as the relative percentage of the geometric mean fluorescence intensity (MFI).

Flow Cytometric Analysis of LDL Uptake. The level of LDL uptake was measured by flow cytometric analysis as previously described.[34] Briefly, cells were seeded as described above and treated with vehicle or xanthohumol for 24 h. After 24 h incubation, the medium was replaced with serum-free DMEM, and BODIPY-FL-LDL (5 μg/mL) (Thermo Fisher Scientific) was added and incubated at 37 °C for an additional 24 h. The level of LDL uptake of 10,000 cells was measured by flow cytometric analysis, and data were expressed as the relative percentage of the geometric mean fluorescence intensity (MFI).

Reverse Transcription Quantitative PCR (RT-qPCR) Analysis. Cells were seeded as described above and treated with vehicle or xanthohumol for 24 h. RNA was extracted from cells using the Total RNA Mini Kit (Geneaid, Taipei, Taiwan). Reverse transcription was performed using the High-Capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed using a reaction mixture containing cDNA, human-specific primers (Table S1), and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). PCR amplification was performed using a Roche LightCycler-480 Real-Time PCR System. The relative differences in expression between groups were analyzed using the ΔΔCt method and normalized with the GAPDH levels in the same samples.

Molecular Docking. To predict the preferable binding sites between LXRα and compounds (T0901317 and xanthohumol), the DOCK module with "Induced fit" refinement in the MOE2015.10 software program was used to perform the molecular docking studies. T0901317 and xanthohumol were manually built in the MOE software package (MOE2015.10) to dock with the LXRα-binding domain (PDB: 3IPQ, LXRα with compound GW3965). The crystal water molecules were removed, and the missing short loops were added

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using MOE software and energy minimized before molecular docking. The scoring used was GBVI/WSA ΔG, a force field-based scoring function that estimated the binding free energy of a ligand to receptor. The preferable binding sites for each compound were determined from the lowest binding free energy, which was the lowest S value of the scoring function.

Statistical Analysis. All experiments were repeated at least three times with consistent results. The data were analyzed using one-way ANOVA with Dunnett's post hoc test, and a p-value of <0.05 was considered statistically significant.

RESULTS

Effect of Xanthohumol on the Viability of HepG2 Cells. Xanthohumol (Figure 1A) is a major prenylated chalconoid from hop plants. To examine the cytotoxic effects of xanthohumol in hepatic cells, HepG2 cells were treated with vehicle (0.1% DMSO) or xanthohumol (5, 10, 20, and 40 μM) for 24 h in DMEM supplemented with 5% lipoprotein-deficient serum (LPDS), and the cell viability was determined using a MTT colorimetric assay. As shown in Figure 1B, xanthohumol (5−40 μM) had no significant cytotoxic effects on HepG2 cells.

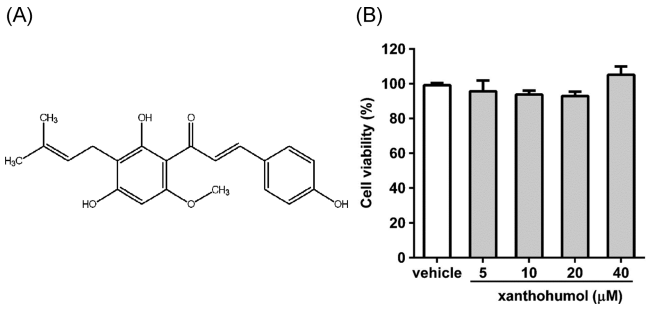

Figure 1. Effect of xanthohumol on viability in HepG2 cells. (A) Chemical structure of xanthohumol. (B) HepG2 cells were cultured in LPDS medium and treated with vehicle (0.1% DMSO) or xanthohumol (5, 10, 20, and 40 μM) for 24 h. Cell viability was measured using a MTT assay. The data represent the mean ± SD of three independent experiments.

Xanthohumol Increases Cell Surface LDLR and LDL Uptake Activity in HepG2 Cells. First, we investigated whether xanthohumol affects the level of cell surface LDLR using flow cytometric analysis. The flow histogram and mean fluorescence intensity (MFI) data showed that cell surface LDLR on HepG2 cells was significantly elevated from 100.0 ± 2.1% to 115.0 ± 1.3% and 135.2 ± 2.7% in 10 and 20 μM xanthohumol-treated cells, respectively, compared to the vehicle group ($p < 0.01$) (Figures 2A and 2B). Moreover, xanthohumol (20 μM) treatment for 24 h markedly enhanced LDL uptake by approximately 40% (from 100.0 ± 0.9% to 139.1 ± 13.2%) compared to the vehicle group ($p < 0.01$) (Figures 2C and 2D). These above results indicated that xanthohumol increases the amount of cell surface LDLR protein and promotes LDL uptake activity in HepG2 cells.

Xanthohumol Increases LDLR Protein but Not mRNA Levels in HepG2 Cells. To address whether the xanthohumol-induced cell-surface LDLR increase is associated with LDLR gene expression in HepG2 cells, the LDLR mRNA and protein expression levels were analyzed using reverse transcription quantitative PCR and Western blot analysis, respectively. As shown in Figure 3A, the LDLR mRNA expression levels in HepG2 cells treated with xanthohumol (10 and 20 μM) were not significantly changed. However, Western

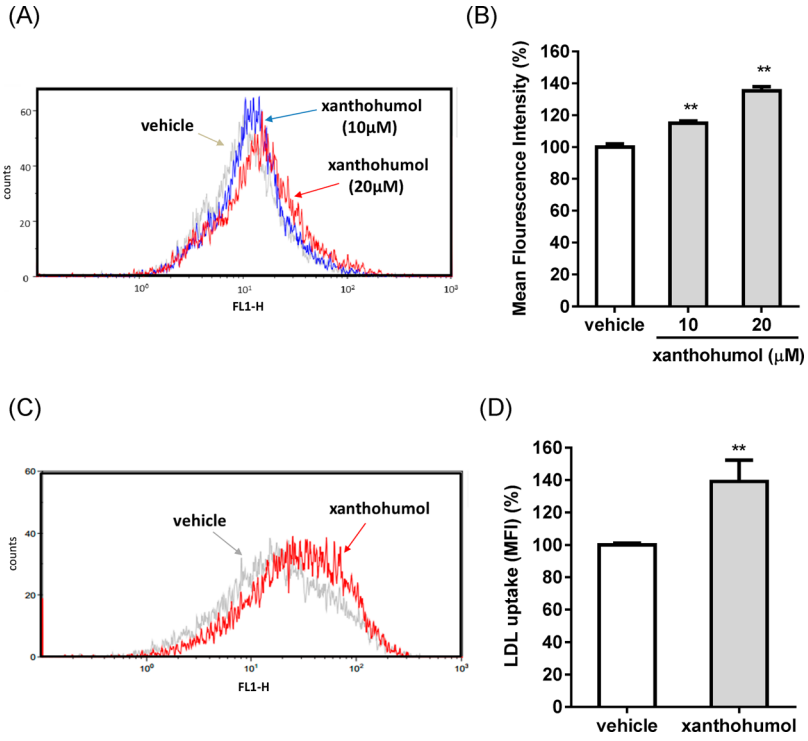

Figure 2. Effect of xanthohumol on the level of cell surface LDLR and LDL uptake in HepG2 cells. HepG2 cells were cultured in LPDS medium and treated with vehicle (0.1% DMSO) or xanthohumol (10 and 20 μM) for 24 h. (A) The level of cell surface LDLR was measured by flow cytometric analysis. A representative histogram of cell surface LDLR level is shown. (B) Summary of cell surface LDLR amount. (C) LDL uptake was measured by flow cytometric analysis. A representative histogram is shown. (D) Summary of LDL uptake. The data represent the mean ± SD of three independent experiments. **$p < 0.01$ represent significant differences compared to the vehicle-treated cells.

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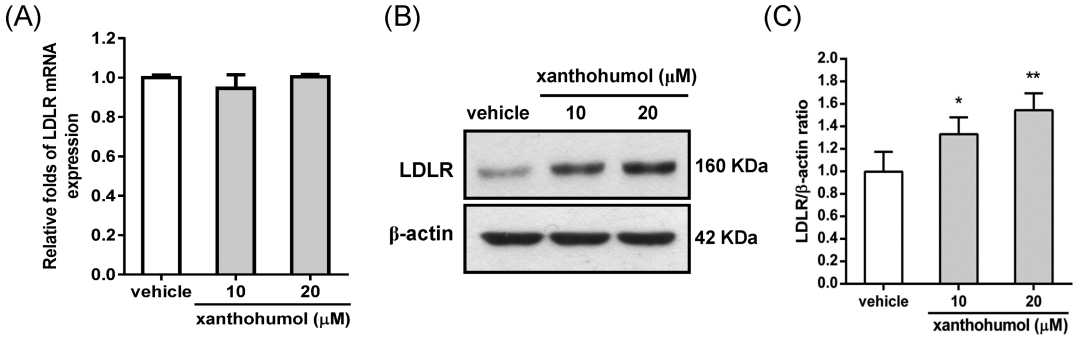

Figure 3. Effect of xanthohumol on LDLR mRNA and protein expression. HepG2 cells were cultured in LPDS medium and treated with vehicle (0.1% DMSO) or xanthohumol (10 and 20 μM) for 24 h. (A) LDLR mRNA was analyzed by RT-qPCR. The values are expressed as the mean ± SD of three independent experiments. (B) The levels of mature LDLR (~160 kDa) and β-actin (~42 kDa) proteins were determined by Western blot analysis. A representative blot is shown. (C) The normalized intensity of LDLR versus β-actin protein represents the mean ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ represent significant differences compared with vehicle-treated cells.

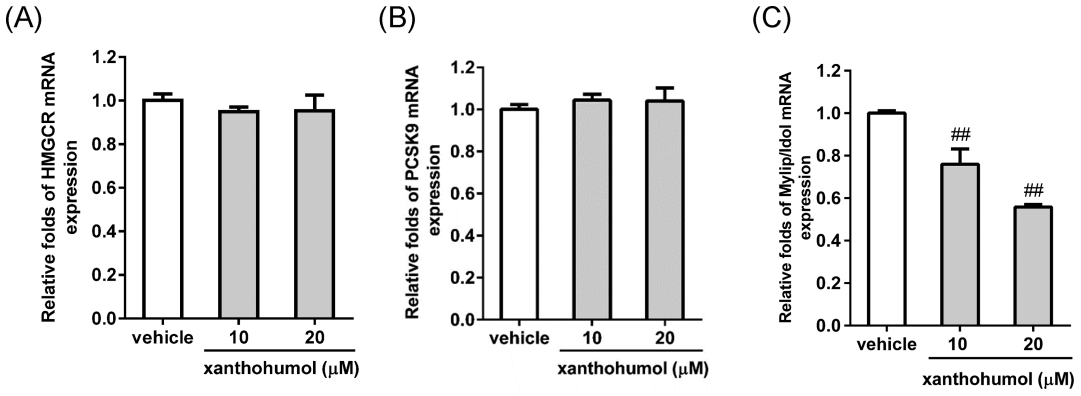

Figure 4. Effects of xanthohumol on HMGCR, PCSK9, and Mylip/Idol mRNA expression. HepG2 cells were cultured in LPDS medium and treated with vehicle (0.1% DMSO) or xanthohumol (10 and 20 μM) for 24 h. (A) HMG-CoA reductase (HMGCR) mRNA, (B) PCSK9 mRNA, and (C) Mylip/Idol mRNA were measured by RT-qPCR. The data represent the mean ± SD of three independent experiments. $^{\#\#}p < 0.01$ represents significant differences compared to the vehicle-treated cells.

blot analysis showed that treatment with 10 and 20 μM xanthohumol significantly increased the level of mature LDLR protein by approximately 1.3- and 1.6-fold, respectively, compared to the vehicle group ($p < 0.01$) (Figures 3B and 3C). These results indicated that xanthohumol enhances LDLR protein levels via post-transcriptional regulation in HepG2 cells.

Xanthohumol Suppresses Mylip/Idol mRNA and Protein Expression in HepG2 Cells. We further determined the effect of xanthohumol on the mRNA expression levels of selected genes for LDLR protein modulation, including HMG-CoA reductase (HMGCR), proprotein convertase subtilisin/kexin type 9 (PCSK9), and myosin regulatory light chain-interacting protein/inducible degrader of LDL receptor (Mylip/Idol), in HepG2 cells. As shown in Figures 4A and 4B, the HMGCR and PCSK9 mRNA expression levels in cells treated with xanthohumol (10 and 20 μM) were not significantly changed. We also demonstrated that the level of PCSK9 protein was not altered by treatment of cells with xanthohumol (20 μM) (Figure S1). However, treatment with 10 and 20 μM xanthohumol significantly suppressed Mylip/Idol mRNA expression by approximately 25% and 45%, respectively, compared to the vehicle-treated group ($p < 0.01$) (Figure 4C).

The level of cell surface LDLR protein is negatively modulated by Mylip/Idol protein in hepatic cells. Therefore, we measured Mylip/Idol protein expression in xanthohumol-treated cells by Western blot analysis. As shown in Figures 5A and 5B, treatment with 10 and 20 μM xanthohumol significantly decreased Mylip/Idol protein expression by approximately 20% and 45%, respectively, compared to vehicle-treated cells. These above results indicated that the xanthohumol-suppressed Mylip/Idol gene expression may be associated with increased LDLR levels in hepatic cells.

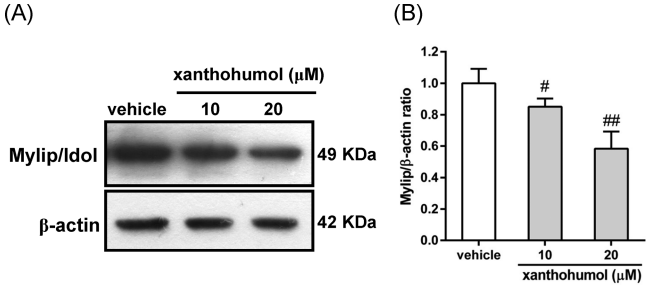

Figure 5. Effect of xanthohumol on Mylip/Idol protein expression. HepG2 cells were cultured in LPDS medium and treated with vehicle (0.1% DMSO) or xanthohumol (10 and 20 μM) for 24 h. (A) Mylip/Idol protein (~49 kDa) was determined by Western blot analysis. A representative blot is shown. (B) The normalized intensity of Mylip/Idol versus β-actin protein represents the mean ± SD of three independent experiments. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ represent significant differences compared to vehicle group.

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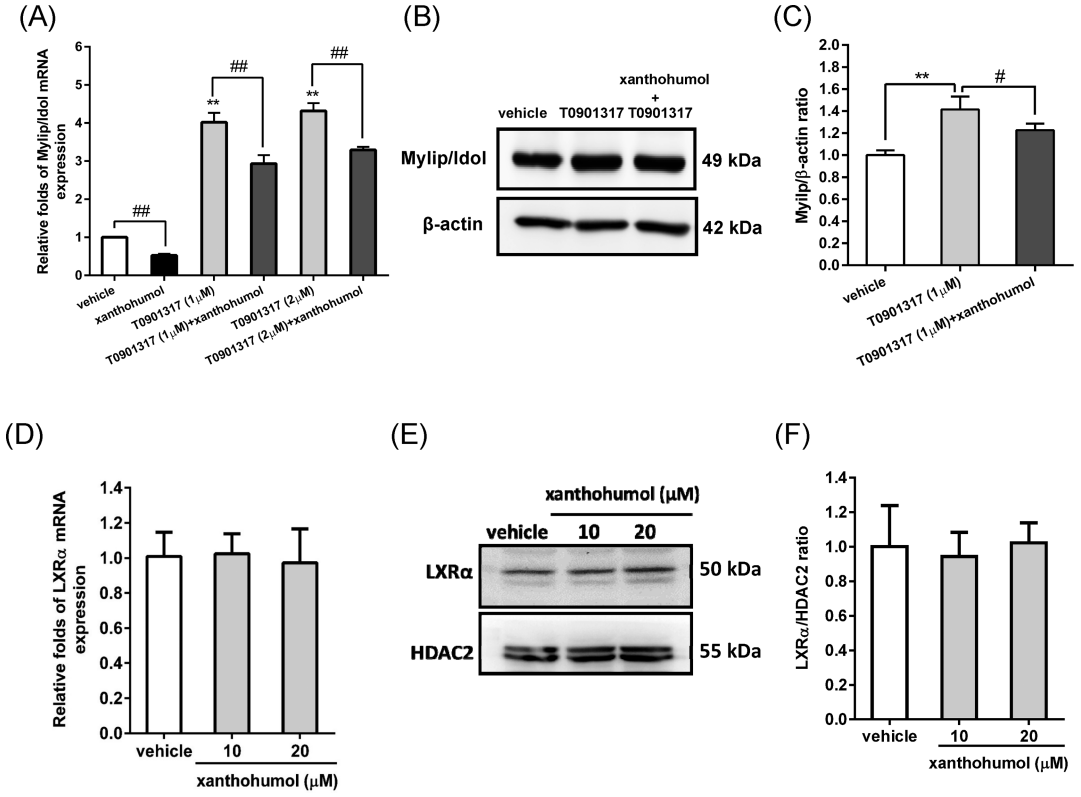

Figure 6. Effect of xanthohumol on LXR-induced Mylip/Idol and LXRα gene expression in HepG2 cells. (A) HepG2 cells were treated with vehicle or xanthohumol (20 μM) for 1 h followed by treatment with T0901317 (1 or 2 μM) for 24 h. Mylip/Idol mRNA was measured by RT-qPCR. The data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ represents significant differences compared to vehicle group. $^{\#\#}p < 0.01$ represents significant differences compared to vehicle- or T0901317-treated cells. (B) The levels of Mylip/Idol and β-actin proteins were determined by Western blot analysis. A representative blot is shown. (C) The normalized intensity of Mylip versus β-actin represents the mean ± SD of three independent experiments. $^{**}p < 0.01$ represents significant differences compared to vehicle group. $^{\#}p < 0.05$ represents significant differences compared to T0901317 alone-treated cells. (D) HepG2 cells were treated with vehicle or xanthohumol (10 and 20 μM) for 24 h. LXR mRNA was measured by RT-qPCR. (E) The levels of nuclear LXRα (~50 kDa) and HDAC2 (~55 kDa) proteins were determined by Western blot analysis. A representative blot is shown. (F) The normalized intensity of LXRα versus HDAC2 represents the mean ± SD of three independent experiments.

Effect of Xanthohumol on LXR-Mediated Mylip/Idol Expression in HepG2 Cells. Mylip/Idol is a direct target for transcriptional activation by liver X receptors (LXRs) in hepatic cells. To investigate the possible mechanism underlying xanthohumol-mediated Mylip/Idol downregulation, the effect of xanthohumol on LXR-induced Mylip/Idol gene expression was examined in hepatic cells. HepG2 cells were pretreated with vehicle or xanthohumol (20 μM) for 1 h followed by treatment for an additional 24 h with the potent LXR agonist T0901317 (1 μM or 2 μM).[35] As shown in Figure 6A, Mylip/Idol mRNA expression was highly induced by T0901317 and reached to 4-fold as compared with vehicle-treated group ($p < 0.01$). While cells were treated with xanthohumol and T0901317, the T0901317 (1 or 2 μM)-induced Mylip/Idol mRNA expression was significantly attenuated by approximately 28% or 24% by xanthohumol, respectively ($p < 0.01$). As shown in Figures 6B and 6C, T0901317 (1 μM) slightly but significantly increased Mylip/Idol protein level approximately to 1.4-fold as compared with vehicle-treated cells ($p < 0.01$). While cells were cotreated with xanthohumol, T0901317-induced increase of Mylip/Idol protein was also slightly attenuated by xanthohumol ($p < 0.05$). LXR-α is an isoform known to be mainly expressed in hepatic cells. Therefore, we further examined the effect of xanthohumol on the levels of LXR-α mRNA and nuclear LXR-α protein in HepG2 cells. As shown in Figures 6D–6F, the levels of LXR-α mRNA and protein were not changed in xanthohumol-treated cells. These data suggested that xanthohumol reduces Mylip/Idol gene expression via counteracting LXR activation in hepatic cells.

Xanthohumol Docks into the LXR-α Ligand-Binding Domain. To investigate whether xanthohumol binds to LXRα to alter LXR activity, the molecular docking program was performed as described in Materials and Methods. As shown in Figures 7A and 7B, the docking results showed that both the T0901317 LXR ligand and xanthohumol docked into the LXRα ligand-binding domain at similar poses. The docking scores were −7.51 and −8.64 for T0901317 and xanthohumol, respectively. These data indicated that xanthohumol has a stronger binding affinity to LXRα than that of T0901317. As shown in Figures 7C and 7D, the ligand−receptor interaction maps show that there are several hydrophobic residues (L331, M298, F326, F315, L260, F335, A261, L428, L435, and W443) around T0901317, indicating that the binding pocket is suitable for lipophilic compound interaction. In addition, xanthohumol was predicted to interact with hydrophobic residues (M298, L260, F257, I339, F335, I313, F326, F315, and A261) and hydrophilic residues (E267, T302, and S264) within the LXRα ligand-binding domain. These data indicated that both xanthohumol and the LXR ligand bind to similar ligand-binding pockets. However, the amino acids for xanthohumol interactions are different from those required for the T0901317 LXRα agonist.

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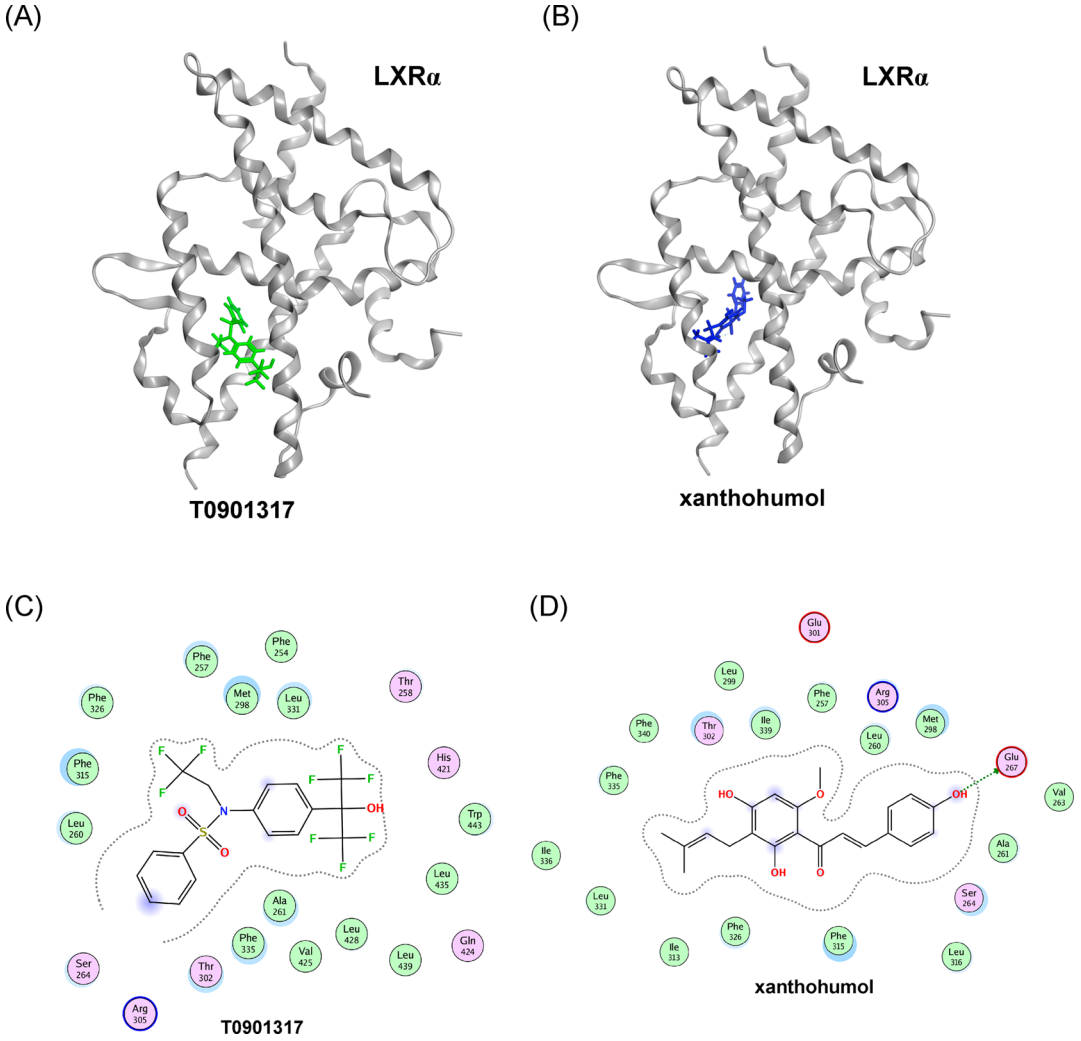

Figure 7. Xanthohumol docks into the LXRα ligand-binding domain. The LXRα receptor is shown in gray ribbon. (A) The best pose of T0901317 docked into the LXRα-binding domain with a docking score of −7.51. T0901317 is shown in green color. (B) The best pose of xanthohumol docked into the LXRα-binding domain with a docking score of −8.64. Xanthohumol is shown in blue color. (C) Two-dimensional T0901317−LXRα interaction map. There are more hydrophobic residues (L331, M298, F326, F315, L260, F335, A261, L428, L435, and W443) than hydrophilic residues (H421 and T302) around the compound. (D) Two-dimensional xanthohumol−LXRα interaction map. In addition to hydrophobic residue (M298, L260, F257, I339, F335, I313, F326, F315, and A261) interaction, the compound also binds into the pocket with hydrophilic residues (E267, T302, and S264) due to more hydroxyl groups in xanthohumol.

Treatment with a Combination of Xanthohumol and Simvastatin Enhances LDLR Activity in HepG2 Cells. Statins, inhibitors of HMG-CoA reductase, are the most effective drugs for reducing plasma cholesterol and have been shown to increase hepatic LDLR amounts and LDL uptake activity. Finally, we investigated the effect of a combination of statin and xanthohumol on Mylip/Idol expression and LDLR activity in hepatic cells. As shown in Figure 8A, simvastatin (1 μM) and xanthohumol (20 μM) significantly suppressed Mylip/Idol mRNA expression by approximately 33% and 35%, respectively, compared to vehicle group ($p < 0.01$). The HepG2 cells that were cotreated with simvastatin and xanthohumol synergistically reduced Mylip/Idol expression by approximately 61% ($p < 0.01$). Furthermore, we demonstrated that a combination treatment of xanthohumol (20 μM) and simvastatin (1 μM) significantly increased LDL uptake compared to xanthohumol or simvastatin alone in HepG2 cells ($p < 0.01$) (Figure 8B). Our results showed that a combination treatment of statins and xanthohumol promotes more Mylip/Idol reduction and effective LDL uptake by hepatic cells.

DISCUSSION

Xanthohumol has been demonstrated to possess the biological abilities for regulation of glucose, cholesterol, and lipid homeostasis in several animal models. In the present study, we demonstrated, for the first time, that xanthohumol markedly counteracts LXR activation, which may result in down-regulation of hepatic Mylip/Idol gene expression, thus elevating LDLR protein levels and LDL uptake activity in HepG2 cells. Furthermore, we predicted that xanthohumol has a higher binding affinity to interact with the LXR ligand-binding domain for modulating receptor activity. Moreover, we demonstrated that a combination of xanthohumol and simvastatin synergistically suppresses Mylip/Idol mRNA expression as well as increases LDLR activity in hepatic cells.

Xanthohumol is a bioactive prenylated flavonoid existing within the flowers of female hops plant with a content of 0.1%−

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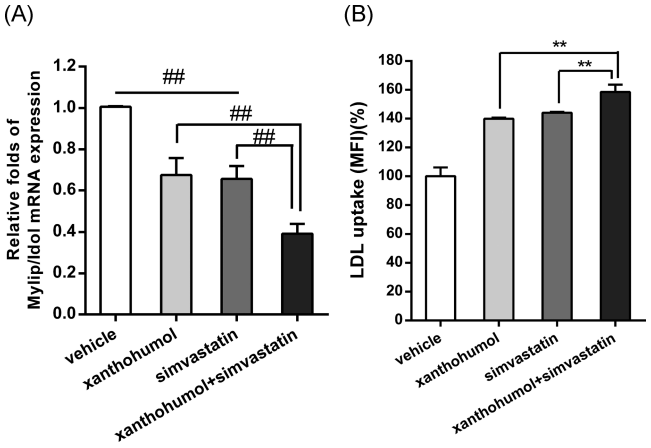

Figure 8. Effect of combination treatment of xanthohumol and statin on Mylip/Idol expression and LDLR activity in HepG2 cells. HepG2 cells were treated with vehicle or xanthohumol (20 μM) for 1 h and then treated with simvastatin (1 μM) for 24 h. (A) Mylip/Idol mRNA was measured by RT-qPCR. The data represent the mean ± SD of three independent experiments. $^{\#\#}p < 0.01$ represents significant differences compared to vehicle-, simvastatin-, or xanthohumol-treated cells. (B) BODIPY-FL-LDL uptake by HepG2 cells was determined by flow cytometric analysis. The data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ represents significant differences compared to the xanthohumol- or simvastatin-treated cells.

1% and an ingredient of beer.[36] Several studies suggested that xanthohumol may exert antiobesity, antihyperlipidemic, and hypoglycemic activities. In animal studies, dietary xanthohumol administration has a low toxicity and is considered to be safe and to have good tolerance.[37,38] The bioavailability of xanthohumol is low in vivo. Legette et al. reported oral administration of xanthohumol (1.86, 5.64, and 16.9 mg/kg body weight) in rats and found that the maximal concentrations (C_{max}) of xanthohumol in plasma were 0.019 ± 0.002, 0.043 ± 0.002, and 0.15 ± 0.01 mg/L, and the bioavailability of xanthohumol was calculated to be approximately 33%, 13%, and 11%, respectively.[39] Metabolism of xanthohumol by gut microflora may be one mechanism for affecting bioavailability.[40] In addition to microorganisms, a rapid accumulation of xanthohumol occurs in intestinal and hepatic cells and most of the intracellular xanthohumol is bound to cellular proteins.[41] Xanthohumol specifically interacting with cytosolic proteins in cells may also contribute to the poor bioavailability in vivo. In vitro studies demonstrated that xanthohumol is spontaneously converted into isoxanthohumol and enzymatically into 6- and 8-prenylnaringenin by hepatic CYP1A2 or gut microorganisms.[42,43] Xanthohumol and its metabolites are conjugated with glucuronides and sulfates.[44,45] Recently, a pharmacokinetic analysis demonstrated that xanthohumol and isoxanthohumol conjugates were major circulating metabolites in human subjects who had received a single oral dose of xanthohumol (20, 60, and 180 mg); however, the 6- and 8-prenylnaringenin were undetectable in most subjects.[46] It was found that the maximal concentrations (C_{max}) of xanthohumol in plasma were 45 ± 7, 67 ± 11, and 133 ± 23 μg/L (equivalent to 0.13 ± 0.02, 0.19 ± 0.03, and 0.38 ± 0.06 μM) and the steady-state plasma concentrations of xanthohumol were calculated to be 1.5, 18, and 40 μg/L (equivalent to 4, 51, and 113 nM) for the 20, 60, and 180 mg doses, respectively. In the present study, we found modulation of LDLR abundance by xanthohumol at concentrations of 10−20 μM, which are used in in vitro cell culture study, though it is higher than what can be achieved in vivo. Our current data showed that xanthohumol at concentrations of 5−40 μM had no significant cytotoxic effects on HepG2 cells. The most abundant dietary source of xanthohumol is beer; however, consumption of regular beer cannot reach pharmacologically relevant concentrations. Xanthohumol enriched functional food or novel formulation may reach pharmacologically relevant concentrations and achieve the concentration used in this study.

Plasma LDL-C reduction has a great impact on decreasing the risk in cardiovascular disorders. The liver is the major organ for cholesterol metabolism, and it regulates circulating LDL-C by its clearance via cell surface LDLRs. Elevating hepatic LDLR expression or its activity results in increased clearance of LDL particles in the circulation and is a successful strategy to modulate cholesterol metabolism.[2] The present study showed that xanthohumol significantly increases cell surface LDLR protein levels and LDL uptake activity in HepG2 cells. Our current findings suggested that xanthohumol possesses potential cholesterol-lowering activity for controlling dysfunctional lipid metabolism. We further demonstrated that xanthohumol markedly increases cellular LDLR protein but does not affect LDLR mRNA expression in HepG2 cells. These findings indicated that xanthohumol upregulates LDLR at the post-transcriptional level. Whether xanthohumol can directly influence the LDLR mRNA stability remains unknown. The stability of LDLR transcripts can be further analyzed in cells treated with xanthohumol followed by the exposure of actinomycin D to interrupt RNA transcription.

PCSK9 and Mylip/Idol are the two main modulators for controlling hepatic LDLR stability at the level of post-translational regulation. PCSK9 and Mylip/Idol proteins share the same LDLR substrate but have distinct mechanisms in regulating LDLR abundance in hepatic cells. Modulation of PCSK9 and Mylip/Idol expression or activity has been considered a potential target for hypercholesterolemia. Extracellular PCSK9, the mature protein form, interacts with cell surface LDLR and triggers receptor-mediated endocytosis, which leads to lysosomal degradation of LDLR. In the present study, we demonstrated that xanthohumol significantly suppresses Mylip/Idol mRNA and protein expression levels; however, PCSK9 expression was not changed in xanthohumol-treated HepG2 cells. Our findings revealed that Mylip/Idol, but not PCSK9, was involved in the xanthohumol-mediated LDLR modulation. The E3-ubiqutin ligase, Mylip/Idol, binds to the cytoplasmic tail of LDLR and promotes ubiquitination in this region, resulting in localization to lysosomes for protein degradation. Overexpression of Mylip/Idol was shown to elevate the ubiquitination level of LDLR, which leads to reduce LDLR protein. It was also found that downregulation of Mylip/Idol by siRNA increased LDLR level and activity.[12] In the present study, we found that Mylip/Idol protein was significantly reduced by xanthohumol in HepG2 cells. We also verified that this Mylip/Idol protein can be specifically detected in HepG2 as well as other cell lines such as Raw 264.7 and Huh 7 cells[47] to confirm the specificity of anti-Mylip antibody used in this study (data not shown). Our findings suggested that xanthohumol-mediated Mylip/Idol reduction may contribute to decrease the level of LDLR ubiquitination and elevate LDLR stability, which lead to enhance LDL uptake in hepatic cells. Whether the reduction in Mylip/Idol induced by xanthohumol directly changes the LDLR ubiquitination or stability needs to be further verified by overexpression or

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knockdown of Mylip/Idol in hepatic cells under treatment with xanthohumol.

Disruption of Mylip/Idol expression results in increased LDLR abundance and LDL uptake rate.[12,19] A loss-of-function Mylip/Idol variant in human individuals has been reported to have low circulating LDL.[48] In the present study, our results suggested that inhibition of Mylip/Idol gene expression by xanthohumol in hepatic cells consequentially increases LDLR level and activity independent of PCSK9 expression. The targeting of Mylip/Idol by xanthohumol may provide an alternative strategy for treatment of hypercholesterolemia and prevention of CHD. It would be interesting to investigate whether xanthohumol acts to downregulate Mylip/Idol in different cells. Recently, Do et al. reported that pro-nerve growth factor (pro-NGF) downregulated Mylip/Idol and increased LDLR levels as well as lipoprotein uptake in neuronal cells.[49] It was also shown that statins enhanced the LDLR and LDL uptake by neuronal cells. Stains were also known to suppress Mylip/Idol expression and may contribute to upregulation of LDLR and LDL uptake in liver cells.[50] Increases in LDLR levels and LDL-uptake activity in neurons may play a critical role in neuronal development and neuroregeneration. Our current findings revealed that xanthohumol markedly suppressed Mylip/Idol expression and exerted increases of LDL uptake activity. Whether xanthohumol may regulate cholesterol metabolism through modulating Mylip/Idol expression in neuronal cells is interesting to investigate in the future.

Mylip/Idol expression is mainly regulated by liver X receptors (LXRs). LXRs, including LXRα and LXRβ, are nuclear receptors of ligand-dependent transcription factors for regulating cholesterol and lipid metabolism.[51,52] LXRα is predominately expressed in the liver and to a lesser extent in macrophages, small intestines, kidneys, and adipose tissues, whereas LXRβ is ubiquitously expressed.[53] LXRα is an essential transcription activator for Mylip/Idol gene expression in hepatic cells. LXR has been reported to activate Mylip/Idol mRNA transcription by binding to LXR-responsive elements (LXREs) within the Mylip/Idol promoter, which contains a consensus direct repeat 4 (DR4) sequence (AGGTCA) separated by 4 nucleotides. HepG2 cells treated with synthetic LXR ligands, such as T0901317, show increased Mylip/Idol expression, resulting in decreases of LDLR protein levels and LDL uptake by cells.[12] These studies demonstrated that the LXR−Mylip/Idol−LDLR axis plays a critical role in the modulation of circulating cholesterol. In the present study, we demonstrated that the LXR ligand, T0901317, significantly induced Mylip/Idol mRNA and protein expression in HepG2 cells, which was consistent with previous studies. The Mylip/Idol mRNA was dramatically induced to 4-fold by T0901317; however, the level of Mylip/Idol protein change was small in T0901317 alone or xanthohumol cotreated cells. Whether the post-transcriptional regulation of Mylip/Idol occurs in LXR agonist-treated cells remains unclear and needs to be further verified. In the present study, the Mylip/Idol mRNA expression induced by T0901317 was significantly attenuated by xanthohumol; however, xanthohumol did not change LXR mRNA and protein expression in HepG2 cells. These results suggested that xanthohumol may serve as a novel LXR modulator and interact with LXR for regulating LXR-mediated Mylip/Idol transcriptional machinery. Our current findings indicate that xanthohumol can counteract the induction in Mylip/Idol mRNA caused by the LXR activation, which in turn modulates the LXR−Mylip/Idol−LDLR axis in the hepatic cells. Because it remains unclear whether xanthohumol is a LXR antagonist or ligand competitor, further studies are required. The precise mechanisms underlying the effect of xanthohumol on the attenuation of LXR activation will be further investigated.

LXR interacts with retinoid X receptor (RXR) to form a heterodimer, which binds to LXRE in the promoter of target genes. In the absence of ligand, LXR/RXR represses gene expression by recruiting corepressors. LXR binding to ligands results in the release of corepressors and recruitment of coactivators to the LXR/RXR heterodimer for activation of target genes.[54,55] The ligand-binding domain of LXRα has been reported to be located at the amino acid residues spanning 206−447. Molecular docking could be used to predict the receptor binding affinity for LXR.[56] In the present study, the molecular docking results predicted that xanthohumol binds to the LXRα ligand-binding domain with a higher binding affinity than the LXRα agonist, T0901317. The key amino acids speculated from the docking results showed that the hydrophobic residues in the binding pocket around xanthohumol or T0901317 were preferable for hydrophobic interaction. In addition to hydrophobic amino acids, some hydrophilic residues within the LXRα-ligand binding domain were predicted to be around xanthohumol and may be involved in receptor interaction via the hydroxyl group in this compound. This docking finding may explain why xanthohumol possesses better binding ability with LXRα than that of T0901317. This study suggested that xanthohumol may bind to the ligand-binding pocket of the LXRα protein, which attenuates LXRα-mediated Mylip/Idol gene transcription in hepatic cells. In the present study, cells treated with xanthohumol alone significantly reduced hepatic Mylip/Idol mRNA levels by approximately 45%, and xanthohumol attenuated T0901317-induced Mylip/Idol mRNA expression by approximately 28% in HepG2 cells. These data implied that the LXR-independent mechanism may also be involved in xanthohumol-mediated Mylip/Idol downregulation in hepatic cells. Recently, a sterol-independent mechanism to regulate Mylip/Idol expression in a LXR-independent manner has been demonstrated.[57] The detailed mechanism by which xanthohumol regulates hepatic Mylip/Idol gene expression via LXR-independent pathway remains to be further clarified.

Statins, HMG-CoA reductase inhibitors, block cholesterol biosynthesis and are commonly used to treat hypercholesterolemia. Statins effectively decrease plasma LDL-C levels and reduce mortality from coronary artery diseases. Statins reduce intrahepatic cholesterol and increase LDLR expression, thus promoting LDL uptake from plasma. However, statin therapy has some limitations for cholesterol management, such as statin intolerance and adverse effects.[58,59] Statins upregulate LDLR and PCSK9 expression through the activation of the SREBP2 pathway. The concomitant PCSK9 expression results in attenuating the effect of LDLR protein and is considered a limitation to the statin efficacy for lowering cholesterol levels.[60] In this study, we demonstrated that a combination of xanthohumol and simvastatin synergistically reduced Mylip/Idol mRNA expression and enhanced the LDL uptake by HepG2 cells. Recently, it was shown that statins also reduced Mylip/Idol expression in liver cells and may contribute to upregulation of LDLR expression.[50] Statins lowered Mylip/Idol mRNA levels and raised PCSK9 mRNA abundance with comparable potencies. Our current findings revealed that

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xanthohumol suppresses Mylip/Idol gene expression and may be used as a potent supplement to statins for hypercholesterolemia therapy.

In conclusion, the results demonstrate the promising hypocholesterolemic effect of xanthohumol in enhancing LDLR abundance and activity via downregulation of Mylip/Idol gene expression in hepatic cells. Our data showed that xanthohumol regulates the LXR−Mylip/Idol−LDLR axis to modulate cholesterol metabolism. Our findings supported that xanthohumol, a phytochemical, may serve as a cholesterol-modulating agent for maintenance of plasma lipid homeostasis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02282.

Primer pairs used in RT-Q-PCR and effect of xanthohumol on PCSK9 protein expression (PDF)

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Notes

The authors declare no competing financial interest.

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